IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Confirmation Number: 8697
Cesare GALLI et al.	Attorney Docket: P66004US0
Serial No. 09/647,939	Group Art Unit: 1632
Filed: December 20, 2000	Examiner: Deborah CROUCH

For: SOURCE OF NUCLEI FOR NUCLEAR TRANSFER

SECOND DECLARATION UNDER 37 C.F.R. § 1.132

1. I, Cesare Galli, am a citizen of Italy and reside at Via Cabrini 12, Cremona, IT 26100, Italy.

2. I am one of the inventors of the above-referenced application.

3. I am familiar with the above-referenced U.S. Patent Application Serial No. 09/647,939 and the rejections raised by the Examiner.

4. I am one of the authors of the paper – Galli et al., Mamalian Leukocytes Contain All the Genetic Information Necessary for the Development of a New Individual, Cloning, Vol. 1, pp. 161-170 (1999) (hereinafter "Clone Paper"); and I am familiar with the content of the Clone Paper.

5. I respectfully submit that the statements relied by the Examiner in the rejection come from the descriptions in the Clone Paper, e.g. page 168, col. 1, parag. 1, lines 11 – 17; page 163, col. 1, lines 3 – 7 and lines 11 – 13; and page 166, col. 1, lines 10 – 14. However, as the author of the Clone Paper, I respectfully submit that, the Examiner has misinterpreted these statements and taken these statements out of context. For example, the following sentences appeared before the statement on page 168, col. 1, parag. 1, lines 11 – 17 – "We are confident that much higher ***future*** rates of embryo development and survival to term will be achieved. This is already clear from an analysis of results obtained by us after the optimization of technical procedures and from ongoing pregnancies" (emphasis added). I respectfully submit that it is clear to a person of ordinary skill in the art that what I call optimization steps apply not to the work of my invention of the present patent application, but to future work and ongoing pregnancies. I respectfully submit herein that the calf of the present invention, Galileo, was not produced with these optimization steps mentioned in the Clone Paper. They have been developed ***later*** to improve the efficiency and they deal with cases for which there were ongoing pregnancies as opposed to Galileo, who was already born.

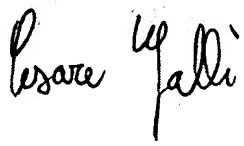
6. The examiner seems to have been persuaded by the work published by Wakayama and Hoechedlinger and therefore doubts that the simple steps set out in the present case are

effective. Wakayama and Hoechedlinger are concerned with mice. They fail to give results without taking special steps. In contrast, Exhibit 1 to this Declaration is a paper published by Lee at el in 2003 (copy enclosed). They discussed complicated cloning procedures which went before them, but report success for pigs in a simple, less labor-intensive whole-cell intracytoplasmic injection. No piezostepper was involved. The pig is closer to the bovine of the examples of the present invention than the mouse. All Wakayama and Hoechedlinger show is that the first part of the invention of the present application – first generation cloning – does not work in mice and when Hoechedlinger added the step of embryo complementation he was successful. The present invention leads to success by including the recloning step.

7. Therefore, the present application has provided sufficient disclosure that enables a person of ordinary skill in the art to practice the invention. Accordingly, withdrawal of the rejection under 35 U.S.C. 112, first paragraph, is respectfully requested.

8. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issuing thereon.

EXECUTED at Cremona this 30th day of January, 2008.



By

Professor Cesare Galli

Enclosure:

Exhibit 1 – Lee et al., Production of Cloned Pigs by Whole-Cell Intracytoplastimic Microinjection, BIOLOGY OF REPRODUCTION 69, pp. 995 – 1001 (2003).

BIOLOGY OF REPRODUCTION 69, 995–1001 (2003)
Published online before print 28 May 2003.
DOI 10.1095/biolreprod.103.015917

Production of Cloned Pigs by Whole-Cell Intracytoplasmic Microinjection[1]

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ABSTRACT

Cloning by somatic cell nuclear transfer has been successfully achieved by both fusing of a donor cell with and injecting an isolated donor cell nucleus into an enucleated oocyte. However, each of the above methods involves extended manipulation of either the oocytes (fusion) or the donor cells (nucleus isolation). Additionally, cloning efficiency can be reduced by low fusion rate of the cell fusion method, and specialized micromanipulation equipment and exacting nucleus isolation techniques are required for the nucleus injection method. Here we report a whole-cell injection technique for nuclear transfer in pigs and the production of cloned piglets with comparable, if not higher, efficiency than the other two nuclear transfer procedures. First, we tested the feasibility of this technique with three types of frequently used donor cells (cumulus, mural granulosa, and fibroblasts) and obtained the optimal nuclear reprogramming conditions for these cells. We further improved our protocol by avoiding ultraviolet exposure during enucleation and achieved a 37% blastocyst rate. We then conducted whole-cell injection using skin fibroblasts from the ear of a sow transgenic for two genes, the porcine lactoferrin and the human factor IX, and produced four live-born cloned transgenic piglets from three recipients. The present study demonstrated the applicability of producing normal, cloned piglets by the simple and less labor-intensive whole-cell intracytoplasmic injection.

assisted reproductive technology, early development, embryo

INTRODUCTION

Pigs are the preferred donors for xenotransplantation, the transplantation of organs from one species to another, and one of the most promising potential solutions to the inadequate supply of human organs. Cloned pigs immunologically compactable to humans can be produced through nuclear transfer by using genetically modified donor cells [1–3].

Two procedures are currently used to produce cloned animals. The cell fusion method, which involves placing a donor cell in the perivitelline space of an enucleated recipient oocyte and fusing the donor and recipient cells with electrical pulses, has been used to generate cloned sheep [4], cattle [5–7], and goats [8] as well as pigs [1–3, 9–15]. Subsequently, a distinctive nonfusion method, in which donor nuclei were isolated and injected into enucleated oocytes by piezo-actuated microinjection, was developed and cloned mice and pigs have been generated [16, 17]. Both of these methods, however, require prolonged manipulation of either the oocytes (fusion) or donor cells (nucleus isolation). These micromanipulation procedures are not only labor intensive but also can reduce the overall cloning efficiency due to the low fusion rate or damage to the isolated nucleus. We have explored alternative methods for the creation of cloned embryos with the aim of increasing the nuclear transfer efficiency and simplifying the cloning procedure. Here we report a new technique involving direct injection of a whole cell into an enucleated oocyte bypassing both the fusion and nucleus isolation processes. Using this new cloning procedure, we produced cloned piglets from the fibroblast cells of an adult transgenic sow.

MATERIALS AND METHODS

Unless stated otherwise, all chemicals for this study were purchased from Sigma Chemical Company (St. Louis, MO).

In Vitro Maturation of Pig Oocytes

Ovaries of prepubertal gilts were obtained from a local slaughterhouse. Oocytes were aspirated from antral follicles (3–7 mm in diameter) and cultured in a 100-μl droplet of maturation medium (BSA-free NCSU23 with 10% porcine follicular fluid, 0.1 mg/ml cysteine, 1% MEM nonessential amino acid, and 0.2 mM pyruvate) with hormonal supplementation (2 μg/ml Follitropin-V, Vetrepharm, Ontario, Canada) in NCSU23 medium at 38.5°C under 5% CO_2 in air for 44 h. In vitro matured oocytes were used in all experiments except for the generation of cloned embryos that were transferred to recipient pigs.

Preparation of Adult Somatic Donor Cells

Fresh cumulus cells were obtained by stripping them off in vitro-matured oocytes in TL-Hepes supplemented with 0.1% hyaluronidase and washing three times in TL-Hepes with 0.4% BSA. Mural granulosa cells were collected during in vitro maturation. Isolated mural granulosa cells were washed with TL-Hepes and then approximately 1×10^7 cells were plated in 60-mm culture dishes (Falcon) containing Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum and 1% antibiotic-antimycotic. Cultures were established by plating cells at a high density, after which they were allowed to reach confluency. To passage cells, confluent cells were disaggregated by incubation in 0.1% trypsin and 0.02% EDTA solution for 1 min at 37°C, and allocated to three dishes. Normally, each cell passage was equivalent to approximately two cell doublings. Fibroblast cell lines were established from skin samples taken from pig ear biopsies. To produce transgenic cloned pigs, fibroblast cells

[1]This manuscript is a scientific contribution (No. 2101) to the Storrs Agricultural Experimental Station at the University of Connecticut. J.-W.L. and S.-C.W. contributed equally to this work.
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Received: 11 February 2003.
First decision: 8 March 2003.
Accepted: 8 May 2003.

ISSN: 0006-3363. http://www.biolreprod.org

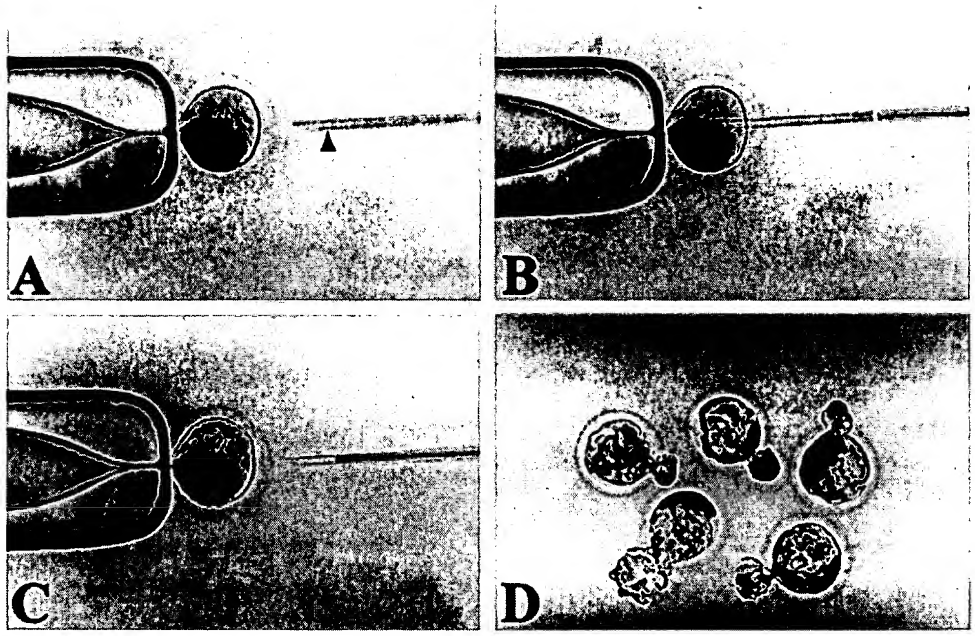

FIG. 1. The whole-cell injection procedure and cloned embryos. **A**) A fibroblast cell (arrowhead) is aspirated into the injection pipette. **B**) The cell is expelled into the cytoplasm of the enucleated oocyte. **C**) Verification of the absence of the donor cell in the injection pipette. **D**) Hatched blastocysts (Day 6) produced by whole-cell injection of fibroblast cells. Original magnification ×100.

were obtained from the ear biopsy of a transgenic sow that expressed two transgenes (αLA-pLF and αLA-hFIX). Briefly, tissue pieces were rinsed in 95% ethanol and placed in PBS supplemented with penicillin (100 IU/ml) and streptomycin (100 μg/ml) and minced into 1- to 2-mm pieces. Approximately five pieces were cultured in 2 ml of DMEM + 10% fetal bovine serum (FBS) and allowed to settle on the bottom of 60-mm tissue culture plates in a humidified 38.5°C incubator with 5% CO_2. Cultures were fed every 5 days and tissue explants were removed and replated every 10 days. The resulting monolayers were harvested by trypsin (0.05%) and EDTA (0.02%) treatment for 5 min. Cells were then passaged 10 times as described above. Granulosa and fibroblast cells were collected after trypsin treatment, frozen in DMEM supplemented with 30% serum and 15% glycerol, and stored at −80°C.

For whole-cell injection, granulosa and fibroblast cells were thawed and cultured to confluency, which normally took 3–4 days. The confluent culture was then extended for 2–3 days without serum starvation. Immediately before whole-cell injection, donor cells were trypsinized, washed by centrifugation, and resuspended in injection medium of TL-Hepes and 10% polyvinylpyrrolidone solution at 1:1. Immunocytochemical analysis for proliferating cell nuclear antigen (PCNA) was conducted in confluent granulosa and fibroblast cells. No PCNA labeling was found in these cells, but nonconfluent granulosa and fibroblast controls were positive. The confluent cells used for whole-cell injection in this study were thus noncycling cells.

Enucleation and Whole-Cell Injection

Recipient oocytes were prepared by centrifugation for 10 min in an Eppendorf centrifuge at 12 000 × *g* in 200 μl TL-Hepes medium to allow detection of the first polar body. Only oocytes with excellent morphology and a visible polar body were selected for this experiment. For enucleation, groups of oocytes were transferred into droplets of TL-Hepes containing 5 μg/ml cytochalasin B (CB), which had previously been placed in the operation chamber on the microscope stage. In the initial experiments, enucleation was accomplished by aspiration of the first polar body and the metaphase II plate in a small amount (<15% of the oocyte volume) of cytoplasm. Successful enucleation was confirmed by examination after staining with 5 μg/ml Hoechst 33342. The enucleation protocol was later improved by partial zona dissection near the polar body and then pressing out cytoplasm at the dissection area [7]. Successful enucleation was confirmed by staining the isolated cytoplasm. Whole-cell injection was conducted using the procedure similar to the intracytoplasmic sperm injection (ICSI) procedure as shown in Figure 1. Briefly, donor cells were transferred to TL-Hepes containing 10% (w/v) polyvinylpyrrolidone and kept at room temperature. A microdrop (10 μl) of injection medium under light mineral oil was placed in the lid of a 60-mm sterile culture dish, which was positioned on an inverted microscope (Olympus, Milville, NY) equipped with micromanipulators (Leica, Bannockburn, IL). Individual fibroblast or cumulus/mural granulosa cells was aspirated into the injection pipette with a sharp, beveled tip (inner diameter 10–12, 15–18, and 20–25 μm) and injected into an enucleated oocyte via the slit that was already made during enucleation process. The cell expelled into the cytoplasm of the oocyte (Fig. 1C) as in ICSI.

Activation of Oocytes

Electrical stimulation was as described by Lee et al. [18] with slight modifications. Briefly, reconstructed embryos were washed and preincubated 20 sec in activation medium (0.25 M mannitol solution supplemented with 0.01% polyvinyl alcohol, 0.5 mM Hepes, 0.1 mM $CaCl_2 \cdot H_2O$, and 0.1 mM $MgCl_2 \cdot 6H_2O$ with pH 7.2) at room temperature. Electrical stimulation was delivered with a BTX Electro Cell Manipulator (Biotechnologies and Experimental Research, Inc., San Diego, CA) to a chamber with two parallel platinum wire electrodes (200 μm outer diameter) spaced 1 mm apart overlaid with activation medium. The reconstructed oocytes were exposed to an electrical pulse for 10 sec at 5 V AC followed by a 1 × 30 μsec pulse at 2.2 kV/cm DC at room temperature. Nonmanipulated, but ultraviolet (UV)-exposed, oocytes were activated 3 h after UV exposure as a control. Following somatic cell injections, oocytes were either immediately activated and then cultured in NCSU23 medium containing 10 μg/ml CB and cycloheximide for 5 h or left in NCSU23 medium at 38.5°C under 5% CO_2 in air for 1.5, 3, and 6 h before electrical activation treatment.

In Vitro Culture of Reconstructed Embryos and Parthenotes

After activation treatments, the reconstructed and control embryos were thoroughly washed and cultured in 50-μl drops of NCSU23 supplemented with 1% MEM nonessential amino acid and 0.4 mg/ml BSA for 7 days at 38.5°C in 5% CO_2 in air without a medium change. The rates of activation, cleavage, and development to blastocyst were examined on Days 2 and 7, respectively, after activation.

Assessment of Whole-Cell Injection and Embryonic Development

To assess successful injection of a donor cell into an enucleated oocyte, the cell membrane was stained with PKH67 green fluorescent cell liner kit (PKH67-GL). When labeled according to the instructions and viewed with a microscope equipped with fluorescein isothiocyanate filter, the membrane of live donor cells fluoresced bright green. To assess chromatin remodeling, oocytes were stained with Hoechst 33342 dye 6, 12, and 24 h after activation. The appearance of swollen nuclei or distinct pseudopronuclei in enucleated cytoplasm was considered evidence of activation.

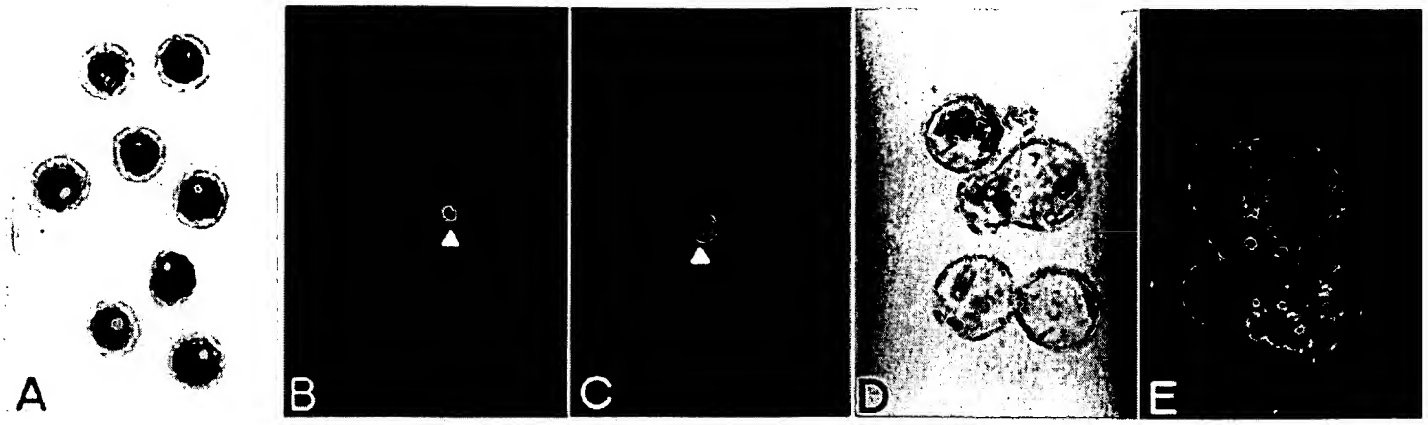

FIG. 2. Nuclear events following whole-cell injection. **A**) Plasma membrane of fibroblast cells was stained with live-membrane fluorescent dye (green). The injected cells were visible in the oocytes' cytoplasm following injection. **B**) The fibroblast nucleus (arrowhead) stained with Hoechst 33342 (blue) was observed 6 h following cell injection at which time the plasma membrane of the donor cell was no longer visible. **C**) Full nucleus swelling (arrowhead) was observed 12 h after oocyte activation. **D**) Hatched blastocysts injected with fibroblast cells. **E**) A hatched blastocyst stained with Hoechst 33342. Original magnification: **A**), ×75; **B/E**), ×200; **C/D**), ×150.

Oocytes, 7 days after activation, were fixed and stained with 5 μg/ml of Hoechst 33342 to assess embryonic development. The cell number for each fixed embryo was counted and its developmental stage recorded.

Superovulation and Embryo Transfer

Pubertal cross-bred gilts aged 8 to 10 mo were synchronized with Regumate (containing 0.4% altrenogest; 20 mg/day; Intervet International B.V, Boxmeer, the Netherlands) mixed in commercial feed and given each morning for 15 days. All donor gilts were injected with 2000 IU pregnant mare serum gonadotropin (PMSG) (Folligon, Intervet) and 80 h later with 1500 IU hCG (Chorulon, Intervet). Recipient gilts were injected with half the dosage of PMSG and hCG administered to the donors. Oocytes were surgically collected 44–46 h after hCG injection by flushing from the oviduct with Dulbecco PBS (Gibco BRL, Gaithersburg, MD). To produce cloned pigs, reconstructed embryos were surgically transferred into the oviducts of synchronized foster mothers 20–24 h after activation. An ultrasound scanner (SSD-500; Aloka, Japan) with an attached 3.5-MHz transabdominal probe was used to check pregnancies 20–21 days after embryo transfer. Pregnant recipients were re-examined by ultrasound around the time of the first to second estrous cycle and again 30 days before the expected due date. Induction of psuedopregnancy in the recipient sows was not conducted.

Detection of Transgene

The umbilical cords and placental tissues were homogenized in 700 μl lysis buffer (50 mM Tris-HCl, 100 mM EDTA, 100 mM NaCl, pH 8.0) containing 500 μg of proteinase K and 70 μl of 10% SDS and were then incubated at 58°C for 16–20 h. Primers, corresponding to αLA-pLF (5′ CCT AGA ACC AAC ACT ACC AG; 3′ AGA AGC CCT CCT TAT GCA GA) and αLA-hFIX (5′ GTG ACC CCA TTT CAG AAT CTT G; 3′ CCG ATT CAG AAT TTT GTT GGC), were employed to amplify 550 bp and 200 bp of respective fragments from the junction region of the transgenes. PCR reactions were performed for 30 cycles with denaturation at 94°C for 30 sec, annealing at 55°C for 1 min, and extension at 72°C for 1 min in a thermal cycler (AG-9600; AcuGen Systems, Lowell, MA). The reaction mixture was then analyzed on a 2% agarose gel, followed by staining with ethidium bromide. The amplified DNA bands were then visualized by ultraviolet transillumination.

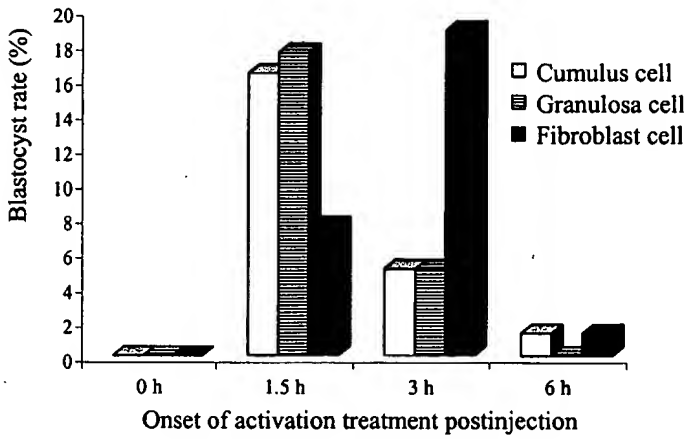

FIG. 3. Blastocyst development of embryos cloned by whole-cell injection of cumulus, mural granulosa, or fibroblast cells and activation at 0, 1.5, 3, and 6 h after whole-cell injection.

Statistical Analyses

Differences in the percentages of oocytes developing to a particular stage were determined by chi-square analysis.

RESULTS

To test the feasibility of cloning by whole-cell injection, we investigated whether, and when, the enucleated oocytes break down the plasma membrane and form a pronucleus from an injected whole cell. We injected fibroblast cells whose plasma membranes were stained with a live-membrane fluorescent dye prior to injection. Immediately after whole-cell injection, the membrane of the injected fibroblast cell was visibly intact and emitted green fluorescence (Fig. 2A). Six hours after whole-cell injection, the plasma membrane (stained green) of the injected fibroblast cell was undetectable, but the nucleus, stained blue by Hoechst 33342, was clearly visible (Fig. 2B). Enlargement of the nucleus was apparent 12 h after activation (Fig. 2C). The injected whole cell was competent to support embryo development to the hatched blastocyst stage in vitro (Fig. 2, D and E).

We then studied the conditioning requirements of the injected whole cells by testing whether an extended time interval between whole-cell injection and oocyte activation would benefit reprogramming and development of the reconstructed embryos. Of particular interest was whether different donor cell types (cumulus, mural granulosa, and fibroblast cells) require different exposure times (0, 1.5, 3, and 6 h) in the cytoplasm of in vitro matured oocytes (Fig. 3). No blastocyst development was observed when activation was conducted immediately after whole-cell injection (0 h), regardless of donor cell types. Activation at 6 h postinjection also produced low blastocyst development for all

TABLE 1. Effects of UV exposure during enucleation on development of cloned and activated oocytes after 7 days of in vitro culture.

Treatment*	No. oocytes injected	No. (%) survived injection	No. oocytes activated	No. (%) oocytes cleaved	No. (%) blastocysts	Cell number (mean ± SEM)
NT + UV	125	115 (92)	105	48 (46)	20 (19)[a]	28 ± 4
NT − UV	125	112 (90)	110	71 (65)	41 (37)[bc]	37 ± 5
Activation + UV	—	—	125	95 (76)	31 (25)[ab]	30 ± 4
Activation − UV	—	—	125	107 (86)	60 (48)[c]	40 ± 5

* Oocytes in the NT + UV group were stained for DNA and enucleated under UV light. Oocytes in the activation + UV group were stained for DNA and exposed to UV light for the same amount of time as those in the NT group.
[a,b,c] Values with different superscripts within columns differ significantly ($P < 0.05$).

cell types tested. The best development was observed when activation was applied at 1.5 h post cell injection for cumulus and mural granulosa cells (18% and 16%) and at 3.0 h for fibroblast cells (19%).

To further improve the development rate of cloned embryos by whole-cell injection, we tested whether removing UV-light exposure from the cloning protocol would significantly improve efficiency. We noticed that UV exposure during oocyte enucleation had a significant and detrimental effect on embryo development. When Hoechst-stained oocytes were exposed to UV during enucleation, blastocyst development by cloned embryos was significantly lower (19%; Table 1) than those produced without UV exposure (37%; $P < 0.05$). The UV exposure also detrimentally affected the development of control parthenogenetically activated oocytes. The blastocyst rate of parthenotes without UV exposure (48%) was significantly higher than those with UV exposure (25%).

After establishing the optimum conditions for cloning by whole-cell injection, we used in vivo matured oocytes for the production of cloned piglets. Fibroblast cells used for whole-cell injection were derived from the ear of a sow carrying two transgenes, pLF and hFIX, both driven by the lactoalbumin promoter (αLA). A total of 685 whole-cell injected oocytes were transferred to nine recipient pigs on Day 1 of the estrous cycle (Table 2). Six of the recipients (67%) were confirmed pregnant by ultrasound 21 days after embryo transfer. Six piglets were aborted from three recipients at Day 23 to 28 of gestation, and four live piglets were born from the remaining three recipients by C-section on February 15, March 23, and April 7, 2002 (Table 2). However, one piglet died 3 days after birth because of infection and abnormal spine development. All live-born (Fig. 4A) and aborted piglets tested positive for both transgenes by PCR (Fig. 4B), which confirmed that they were derived from the donor sow.

DISCUSSION

In the present study, we have shown for the first time that directly injecting a whole cell into the cytoplasm of an enucleated pig oocyte is feasible for the production of cloned embryos and piglets. The method of whole-cell injection for nuclear transfer had not been attempted previously because of concerns that the plasma membrane of the donor cell may persist in the oocytes resulting in failure to release the nucleus. In the present study, we used live dye staining of the donor cell membrane and observed the process of plasma membrane dissolution. The membrane of the majority of the donor cell dissolved relatively rapidly, within an hour of injection. However, the green fluorescence was visible in some oocytes up to 6 h before becoming undetectable, suggesting that the plasma membrane can persist in oocytes for relatively long periods of time. It is unlikely that the disappearance of the fluorescence in the later group resulted from dye diffusion because the dye persisted more than 24 h in stained cells left in the culture medium (J. Lee and X. Yang, unpublished observation). However, the exact mechanism for the donor cell's plasma membrane dissolution in the oocyte is unclear. Two possible mechanisms can potentially explain the dissolution process. First, the oocyte might have actively recognized the donor cell's plasma membrane or its surface proteins as belonging to a cell surface, not to the cytoplasm of the oocyte. This recognition would then lead the oocyte to actively degrade the donor cell's plasma membrane or transport it to the oocyte's cell surface. This active dissolution would then release the nucleus for reprogramming.

On the basis of previous studies on plasma membrane receptor recycling, we consider the above-mentioned mechanism plausible. Mammalian cells have complex sorting systems that recognize cellular membranous vesicles bound for the intracellular organelles or the plasma membrane through protein molecules embedded on the membrane of these vesicles. These vesicles are then transported to the appropriate locations [19]. A good example of intracellular plasma membrane vesicles that are recognized and transported back to the surface is the low-density lipoprotein (LDL) receptor recycling process [20]. It has long been known that the surface membrane engulfed during internalization of the membrane-embedded LDL receptor is recycled back to the surface by this membrane/protein recycling/sorting system [20]. In the case of whole-cell injection for nuclear transfer utilized in our study, it is possible that the oocyte was able to recognize the plasma membrane of the donor cells as being ectopic. Therefore, it actively dissolved the membrane of the whole cell and recycled it to the oocyte's surface membrane.

The second possible mechanism for donor cell membrane dissolution may be that the donor cell membrane was damaged during whole-cell injection and the leaky plasma membrane then released the nucleus, which resulted in its reprogramming. This mechanism, although possible, is less likely and may account for only a small portion of all donor membrane dissolution in the whole-cell injection procedure. This is because if the oocyte does not recognize the plasma

TABLE 2. Embryo transfer and pregnancy rates of whole cell-injected embryos cloned from skin fibroblasts from a double transgenic sow (αLA-pLF and αLA-hFIX).

No. oocytes collected	1036
No. (%) oocytes enucleated	893 (83)
No. (%) oocytes injected	801 (77)
No. (%) embryo cultured	718 (69)
No. (%) embryo transferred	685 (66)
No. (%) recipients pregnant/total	6/9 (67)
No. recipients farrowed	3
No. (%) cloned piglets born	4 (0.4)

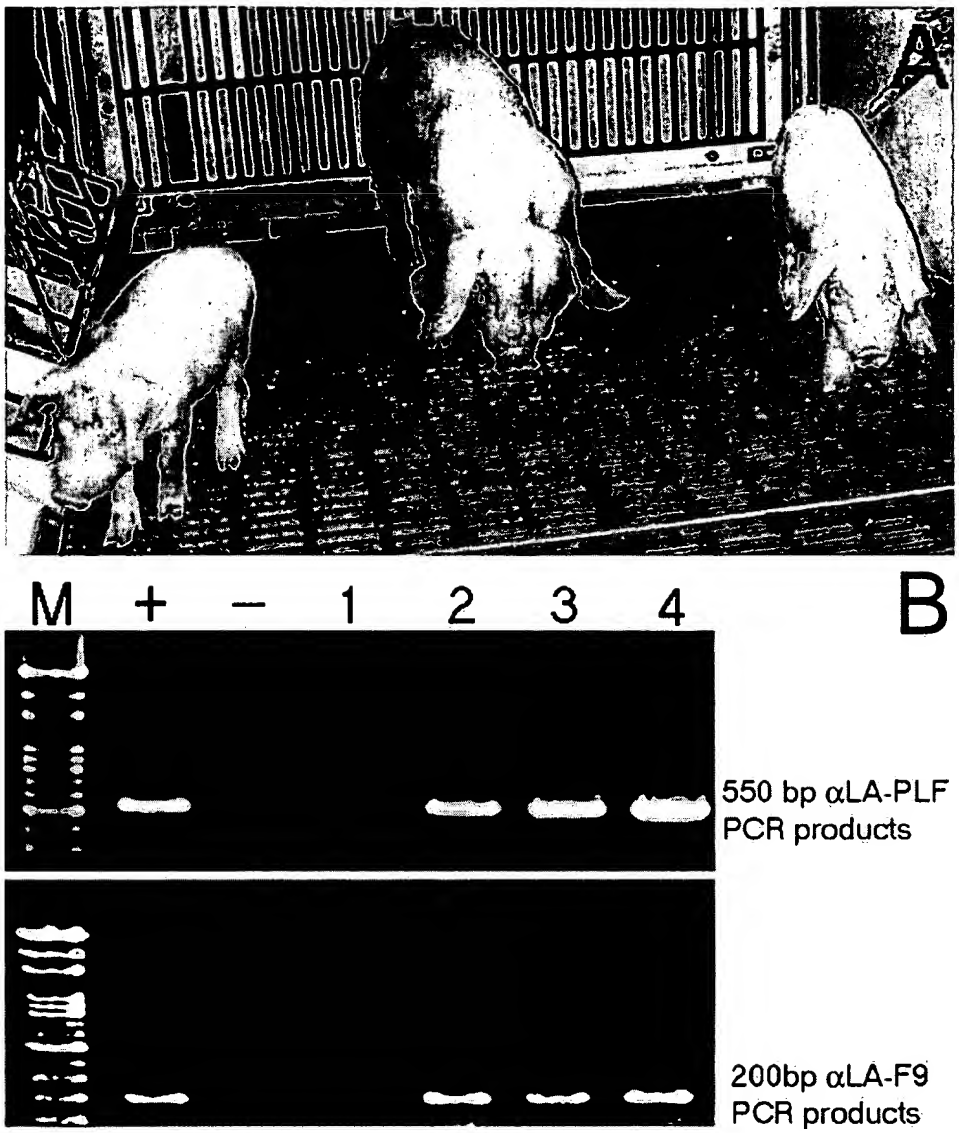

FIG. 4. Piglets born from cloned embryos generated by whole-cell injection of transgenic fibroblast cells. A) Three cloned piglets born from three recipients on February 15, March 23, and April 7, 2002. B) A representative PCR assay for the αLA-pLF and αLA-hFIX double transgenes. Expected fragments of 550 bp for pLF and 200 bp for hFIX were obtained. Lane 1, recipient pig; lane 2, placenta of a cloned fetus; lane 3, umbilical cord of a cloned fetus; lane 4, donor cells. +, positive control; −, negative control; M, 100-bp markers.

membrane of the injected whole cell as being ectopic, it will likely treat the whole cell as an intracellular organelle with membrane damage. The oocyte may then actively repair the membrane damage, in which case the whole-cell injection would not have succeeded or had a much reduced success rate. Our results in which whole-cell injection produced a high rate of blastocyst development, suggested that the plasma membrane damage, if any, was not repaired. Instead, the plasma membrane was eventually dissolved. These results argue against the possibility that donor membrane damage is the only mechanism for its dissolution, and they argue for the possibility that the oocyte was able to distinguish membranes of the donor cell and those of the intracellular organelles. Experiments to test the plasma membrane recycling hypothesis are currently being conducted. Regardless of the mechanism, our results that cloned embryos and piglets were produced by the whole-cell injection method demonstrated that the oocyte is capable of dissolving the plasma membrane and that the whole-cell injection procedure, bypassing the cell fusion or nucleus isolation procedures, represents a feasible new procedure for efficient production of cloned embryos requiring less oocyte or donor cell manipulation.

In the present study, we found that different donor cell types require different amounts of conditioning time in the oocytes before activation. Fibroblast cells required longer exposure to the MII cytoplasm than granulosa cells when whole cells were used for injection. These results are consistent with those in mice and pigs showing that delayed activation for fusion as well as nucleus injection methods was beneficial to embryo development [12, 16]. Our results point to the possibility that the plasma membrane of different donor cell types requires different amounts of time for dissolution. This phenomenon is consistent with the observation that different fusion rates are associated with fibroblast and cumulus cells in the cell fusion method of cloning (X. Yang, unpublished observations). Both the differences in fusion rate and conditioning time may be attributable to the differences in the membrane properties of these cells such as protein and/or lipid compositions.

Since the first success of cloning in pigs was reported [9], there have been another 10 reports in which a live cloned piglet(s) was successfully generated [1–3, 10–15, 17]. Among these reports, the fusion method was employed in all studies except that of Onishi et al. [17], who utilized the nucleus injection method. Blastocyst development by the fusion method, in reports in which in vitro development data were available, was relatively low, up to 10% with

either in vivo or in vitro matured oocytes [10, 12–14] with the exception that recently Boquest et al. [11] reported a 23% blastocyst rate by cloning with in vivo matured oocytes. For the nucleus injection method, Onishi et al. [17] obtained as high as 31.2% blastocyst development by using in vivo matured oocytes. However, this method of nucleus injection requires specialized micromanipulation equipment and nuclear isolation techniques.

In the present study, by using in vitro matured porcine oocytes and the simple whole-cell injection technique and conventional micromanipulation equipment, we obtained an in vitro blastocyst development rate as high as 37%. We attribute the high efficiency of our new cloning procedure to the following: 1) our technique for the injection of whole cells reduced the manipulation time of donor cells and recipient oocytes as compared with the other two nuclear transfer methods and therefore is beneficial to embryo development; 2) the injection of a whole cell assured delivery of all cellular components to the enucleated oocytes; these components of the donor cells might be important for later development in pigs; the microtubule-organizing center, for example, is needed during natural fertilization in most mammals except for the mouse [21]; and 3) whole-cell injection assured delivery of DNA into each injected oocyte and thus avoided fusion failure and potential damage to the nucleus during isolation. Other conditions that are not unique to the whole-cell injection technique yet helped improve our nuclear transfer efficiency may be that we avoided UV exposure and the use of Hoechst staining during enucleation, which was a common practice for cloning [10, 22] and was found to cause abnormal meiosis and poor development [23–26].

In addition to the high in vitro development rates of cloned embryos, we also obtained a high pregnancy rate (67%). Even with a relatively small number of embryos transferred into each recipient (70–80 embryos vs. 150–250 embryos/recipient in most previous reports), we obtained an overall efficiency comparable with the other two cloning techniques in producing cloned piglets.

In summary, our whole-cell injection procedure is less labor intensive, requires no special micromanipulation equipment, and is as efficient in the generation of cloned pigs.

ACKNOWLEDGMENTS

We are grateful to TransOva Genetics (Hull, Iowa), especially Dr. Dave Faber for his support and Mr. Jerry Pommer for his coordination. We also thank the administrators, staff, and scientists at the Animal Technology Institute in Taiwan: Dr. J.H. Lin for C-sections; Dr. C.N. Weng and P.C. Yang for their support; W.D. Fang and S.T. Liu for taking care of cloned piglets; and Mr. C.J. Chou, Miss W.T. Lien and S.Y. Hwang for their help on the cloning work. The authors would also like to thank M. Julian for her critical reading and comments on the manuscript.

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